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Principal Investigator: John W. Frost

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Draths Corporation
2367 Science Parkway, Ste. 2
Okemos, MI 48864

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Green Synthesis of D-1,2,4 – Butantetroil from D-Glucose

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Technical Report

a. Scientific and Technical Objectives

The current microbial synthesis of D-1,2,4-butanetriol is based on the use of D-xylose as the starting material. Although D-xylose is abundant in hemicellulose, streams of D-xylose sufficiently pure to support microbial growth are not available in the U.S. Because of the current expense of D-xylose, a microbial synthesis of D-1,2,4-butanetriol from D-glucose was targeted for development. The activity of *mdlC*-encoded benzoylformate decarboxylase is essential to improving the yields and concentrations of microbe-synthesized D-1,2,4-butanetriol. Codon optimization of *Pseudomonas putida mdlC* for heterologous expression in an *Escherichia coli* host was therefore examined.

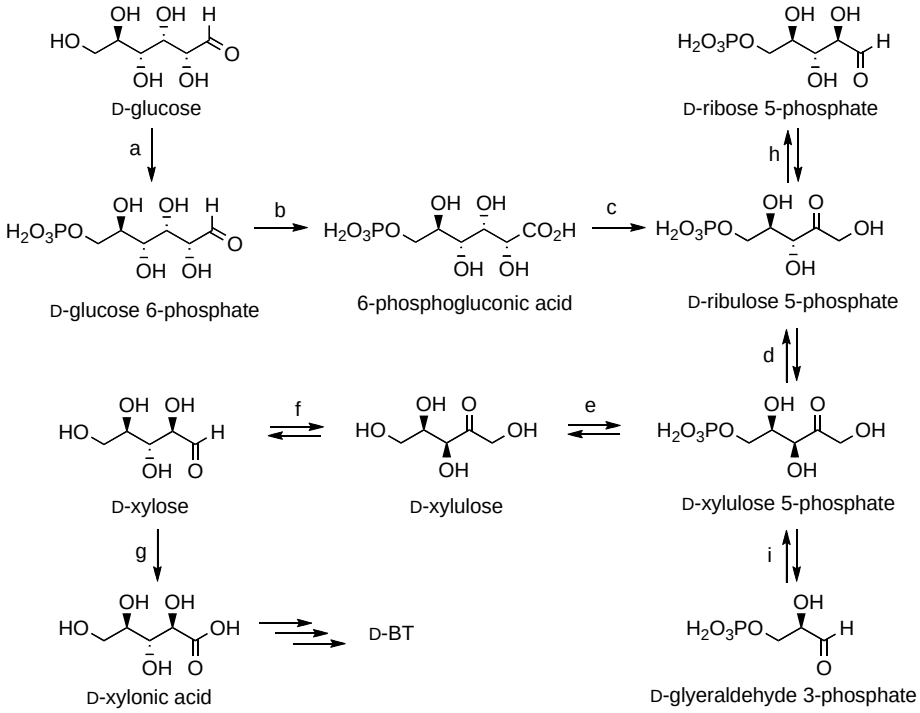

Figure 1. Microbial Synthesis of D-Xylonic Acid from D-Glucose. (a) carbohydrate phosphotransferase (*ptsG*, *crr*); (b) D-glucose 6-phosphate dehydrogenase (*zwf*); (c) 6-phosphogluconate dehydrogenase (*gnd*); (d) D-ribulose phosphate epimerase (*rpe*); (e) phosphatase; (f) D-xylulose isomerase (*xylA*); (g) *C. crescentus* D-xylose dehydrogenase (*xdh*); (h) D-ribose 5-phosphate isomerase (*rpiA*, *rpiB*); (i) transketolase (*tktA*, *tktB*).

b. Approach

A required first step to assemble an *E. coli* construct capable of synthesizing D-1,2,4-butanetriol from D-glucose is to create a pathway for conversion of D-glucose into D-xylonic acid (Figure 1). The isozymes of D-ribose 5-phosphate isomerase encoded by *rpiA* and *rpiB* and the isozymes of transketolase encoded by *tktA* and *tktB* in *E. coli* were the focus of research activities. Inactivations of various combinations of these loci were examined in constructs where *yfbT*-encoded sugar phosphatase activity was overexpressed. To increase the activity of *mdlC*-encoded benzoylformate decarboxylase, the codons for this *P. putida* geme were optimized in

silico for expression in *E. coli*. Codon-optimized *mdlC* was chemically synthesized and the impact of codon optimization on the specific activity of heterologously expressed benzoylformate decarboxylase examined in *E. coli* cultured under fermentor-controlled conditions.

c. Concise Accomplishments

Triple knockouts *E. coli* WY9 (*tktA rpiA rpiB*)/pWY1 synthesized 2 g/L of D-xylonic acid from D-glucose, and *E. coli* WY11 (*tktA tktB rpiA*)/pWY1 synthesized 1 g/L of D-xylonic acid from D-glucose. Codon-optimized *P. putida mdlC* heterologously expressed in an *E. coli* host used for synthesis of D-1,2,4-butanetriol resulted in higher specific activities of benzoylformate decarboxylase relative to heterologous expression of wild-type *mdlC* in the same *E. coli* host when these constructs were cultured under fermentor-controlled conditions.

d. Expanded Accomplishments

Conversion of D-glucose into D-xylonic acid (Figure 1) required at least partial inactivation of D-ribose 5-phosphate isomerase, which is encoded by *rpiA* and *rpiB*, and partial inactivation of transketolase, which is encoded by *tktA* and *tktB*. Most of the D-ribose 5-phosphate isomerase activity in *E. coli* is encoded by *rpiA*, while most of the transketolase activity is endoced by *tktA*.

Table 1. Summary of genetically modified *E. coli* strains.

KIT22	W
KIT25	W
KIT29	W
WY1	W
WY2	W
WY5	W
WY6	W
WY8	W
WY9	W
WY10	W
WY11	W

E. coli hosts with multiple gene knockouts were constructed using a combination of the Wanner methodology and phage P1 transduction. *E. coli* KIT19 was generated directly from wild-type W3110 strain by homologous recombination of the chloramphenicol resistance gene to replace the chromosomal *tktA* gene. The mutation of KIT19 was first transduced into WN7, followed by curing the drug resistance using flipase-mediated recombination to obtain KIT25. Strain WY1 was generated by recombination of a fragment containing the kanamycin resistance gene homologous to the *serA* to *rpiA* genomic region into host KIT25. A similar flipase-mediated recombination was then carried out to cure kanamycin resistancy in WY1 to generate WY2. KIT29 was derived from WN7 by replacing the genomic copy of *xylB* gene with a *xdh*-Cm^R gene cassette. The *xylB* gene encodes D-xylose kinase and the chromosomal modification on KIT29

abolished the native *E. coli* ability to utilize D-xylose as a sole carbon source for growth. *C. crescentus xdh* gene encoding D-xylose dehydrogenase is expressed under the control of the *xylA* promoter. *E. coli* WY5 was produced by phage transduction of the mutation in KIT29 into strain WY2. Each of the *E. coli* strains KIT20 and KIT22 contain genomic single gene knockout of *tktB* and *rpiB* gene, respectively, and were used as a donor strain to further modify WY5 genome. Phage transduction of KIT22 into WY5 afforded WY6. Flipase-mediated recombination of WY6 cured both the kanamycin and chloramphenicol resistance genes in WY6 and afforded strain WY9. On the other hand, flipase-mediated recombination of strain WY5 removed the kanamycin resistence gene and afforded strain WY8. Phage transduction of KIT20 into WY8 afforded WY10. Flipase-mediated recombination of WY10 removed the chloramphenicol resistance gene and afforded strain WY11.

E. coli WY9/pWY1(*yfbT*) and *E. coli* WY11/pWY1(*yfbT*) were evaluated for conversion of D-glucose by growth in LB-xylose medium, induced with addition of IPTG, harvested and the cell pellet washed thoroughly to remove any residual D-xylose. Resuspension of *E. coli* WY9/pWY1(*yfbT*) in LB-glucose resulted in the synthesis of 2 g/L of D-xylonic acid while similarly cultured *E. coli* WY11/pWY1(*yfbT*) synthesized 1 g/L of D-xylonic acid.

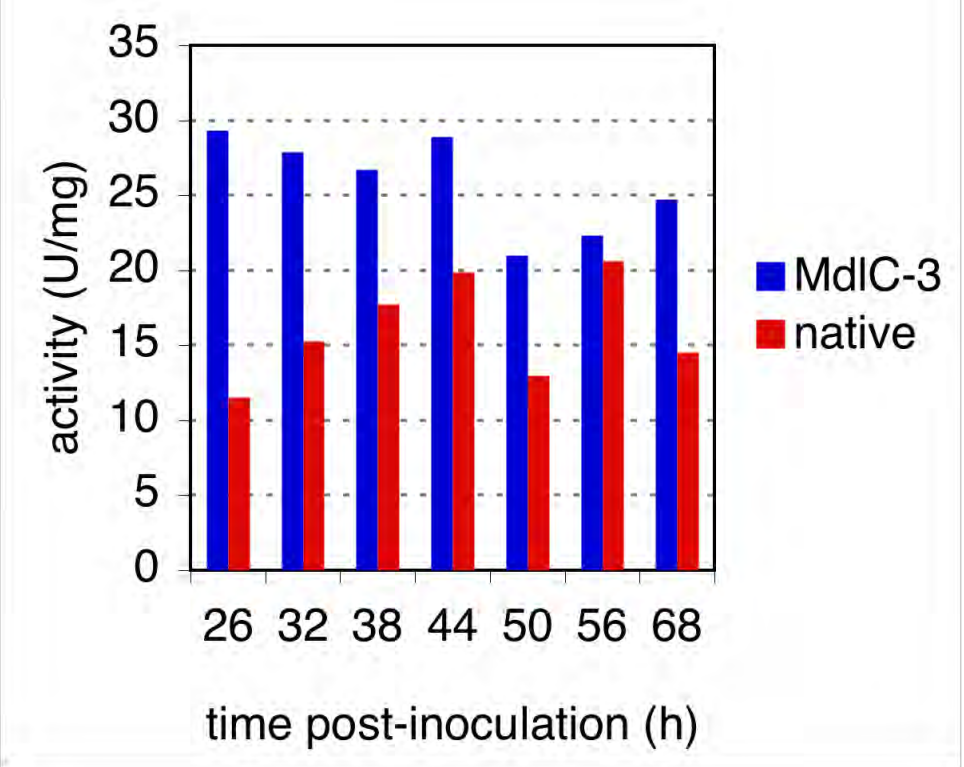

Figure 2. Comparison of Codon-Optimized with Native MdlC. Specific activity (μmol/min/kg) of plasmid-localized, codon-optimized *mdlC*-3 relative to plasmid-localized native *mdlC* in lysates of fermentor cultivated cells.

Codon optimization of GC-rich *P. putida mdlC* for expression in *E. coli* employed an algorithm available through DNA 2.0, which was also the company that synthesized the codon-optimized *mdlC*. Plasmid-localized, codon-optimized *mdlC* and plasmid-localized, native *mdlC* were

expressed in *E. coli* KIT18 (W3110*serAΔyjhHΔyagExylAB*::*xdh-adhP-P*$_{tac}$*ΔyiaEΔycdW*) and cultured under fermentor-controlled conditions. (NOTE: KIT18 has been used as a host for synthesis of 18 g/L of D-1,2,4-butanetriol in 54% (mol/mol) yield from D-xylose under fermentor controlled conditions.) As seen in Figure 2, codon-optimized *mdlC* gave almost a three-fold higher MdlC specific activity relative to native *mdlC* at 26 h after inoculation. Codon-optimized *mdlC* also maintained a substantially higher specific activity over the entire course of the fermentor run relative to native *mdlC*.

e. Work Plan

With the successful conversion of D-glucose into D-xylonic acid, attention will now focus on synthesis of D-1,2,4-butanetriol from D-glucose. The specific activity of *mdlC*-encoded benzoylformate decarboxylase will be further improved and decarboxylases that are more active with 3-deoxy-D-*glycero*-pentulosonic acid as substrate are to be identified.

f. Major Problems/Issues

None.

g. Technology Transfer

h. Foreign Collaborations and Supported Foreign Nationals